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Original Paper

Assessment of Microbial Air Quality of Nashik City with Particular Reference to *Mucorales* Fungi, and *in Vitro* Evaluation of Two Triazole Antifungal Drugs against the Prevalent *Mucor* Species

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Abstract

Air pollution particularly that of particulate matter (PM 2.5, PM 2.10), carbon monoxide, ozone, nitrogen dioxide, sulfur dioxide, ammonia, lead, and air microbial contaminants, has serious consequences on human health. Air pollution in metros and cities around the world is measured for the above parameters except for the microbial air contaminants. However, microbial air contaminants are important sources of microbial infection in humans and particularly airborne fungi are known to cause diseases like Aspergillosis and Mucormycosis in immunocompromised patients which are about 160 million in the world.

In the year 2021, Mucormycosis disease was reported as a post-covid infection in several states of India as a fatal disease caused by a black fungus (Mucor) prevalent in the atmospheric air. In the present study, we assessed the microbial air quality (colony forming unit of microbes/m^3 of air) of Nashik city air, in India, for its microbial contaminant, particularly Mucor sp., and further the prevalent Mucor sp. was evaluated for its reaction to two triazole antifungal drugs viz. Itraconazole and Fluconazole available in medical stores.

The air quality index of 90 CFU/tidal volume for Mucor species was regarded as safe, based on the studies. Both the triazole drugs at their active ingredient concentration (1000 µg/mL) were unable to check the growth of Mucor fungi. The paper discussed in detail the methods for enumeration of microbial contaminant/m^3 of air and in tidal volume.

Keywords

microbial air pollutant, Mucor, mucormycosis, triazole antifungal drugs, microbial CFU/tidal volume, microbial AQI

1. Introduction

Mucormycosis, as a post-covid infection was a serious health issue in many Indian states in 2021 (Borkar, 2021). The fungus responsible for this disease was a species of *Mucor*, which is mainly present in the air. Based on the anatomic localization of infection by *Mucor*, mucormycosis can be classified as one of 6 forms *viz.* rhinocerebral, pulmonary, cutaneous, gastrointestinal, disseminated, and uncommon presentation (Petrikkos et al., 2012). The infection of mucormycosis has serious implications with permanent disabilities like debridement of the eyes and jaws of the patients, and even death due to invasive infections (Neilstone et al., 2021). Generally, the infection of mucormycosis was observed in post-covid patients, with lowered immunity, or in immunocompromised patients (Pak et al., 2008). The specific immunocompromised conditions include: 1) severe immunocompromised (non-HIV)-active leukemia, lymphoma, generalized malignancy, aplastic anemia, graft versus host disease, congenital immunodeficiency, solid open transplant or bone marrow transplant within 2 years of transplantation, or persons whose transplants are of longer duration but who are still taking immunosuppressive drugs. 2) Chronic diseases with limited immune deficits-asplenia, chronic renal disease, chronic hepatic diseases (cirrhosis and alcoholism), diabetes, nutritional deficiencies, and people affected by pandemic diseases like covid-19 (Monica & Chandraprabha, 2022).

At present 2% of the world population, i.e., about 160 million people are reported to be immunocompromised in the world (Anonymous, 2022) and probably the targeted population for the diseases like mucormycosis caused by *Mucor* fungus present in the atmospheric air.

Although the source of *Mucor* infection is through the air, which we breathe, no documentation for this fungus in the City Air quality Index around the world, is available on regular basis in the metros. The air pollution in many metros and cities around the world is measured for particulate matter (PM 2.5 and PM 10), Carbon monoxide (CO), and other harmful gases, but nowhere it is depicted for fungal air contaminants responsible for human diseases like Aspergillosis (Borkar, 2020) and Mucormycosis (Borkar, 2021). The lack of air quality studies for harmful air microbes can be seen as a setback to keeping in check the source of infection and the optimal population threshold of the microbes in the air for human fungal infection particularly mucormycosis.

Therefore, in the present study, we assessed the air quality of Nashik city air, in India, for its microbial contaminant particularly *Mucor* sp. a known fungus to cause Mucormycosis in humans. The Mucormycosis fungi are also reported to develop resistance to the antifungal drug Amphotericin-B, the most commonly used drug for its control (Ellis, 2002; Asghar, 2019). Therefore, we evaluated two triazole antifungal drugs for their effectiveness against the *Mucor* fungus prevalent in the Nashik city air sample.

2. Method

2.1 Detection of Microbial Flora in the Air Sample

2.1.1 Growth Medium Used for Detection of Microbial Flora in Air Sample

Potato-dextrose-agar (PDA) medium was used for the detection of microbial flora in the air sample. Petri plates of 7.5 cm diameter having sterilized PDA media were exposed to air breeze at 10 different locations in the city jurisdiction of Nashik Municipal Corporation. These 10 locations represented the east, west, north, south, and center locality of the city. Petri plates in 3 replicates/locations were used to assess the prevalence of microbes in the air at a given location.

The air sample for microbial prevalence was assessed in the 2nd week of June 2022. The maximum temperature, minimum temperature, and atmospheric humidity during the sampling period were 35^{0}C, 29^{0}C, and 77% respectively.

The air sample was assessed for the microbial population of *fungi*, *bacteria*, *actinomycetes*, *yeast,* etc., and, particular emphasis was given to the presence of Mucorales fungi in the air, as during the same period in 2021, the Mucorales fungi (*Mucor* sp. or black fungus) caused Mucormycosis disease in the city patients.

2.1.2 Collection of Air Samples on Microbial Growth Media

The microbial growth media plates were exposed to the city air of the respective location for 1-2 minutes and covered with an upper lid. Such exposed plates for each location were numbered and brought to the laboratory within a period of 3 h. These plates were incubated in a BOD incubator at 29 ±1^{0}C temperature for the growth of airborne microbes trapped on the plates. Reading for the growth of bacterial colonies was taken after 48 h of incubation while reading for the presence of fungal colonies was noted after 3 days onwards and up to 10 days.

2.1.3 Enumeration of Microbial Colony Forming Units (CFU)/m^3, Trapped in the Media Plates

To enumerate the Colony Forming Unit (CFU) of each type of microbe on the growth media in a plate of 7.5 cm diameter, the simplified method was followed. The 7.5 cm diameter of the plate was earmarked length and breadthwise, as shown in Figure 1, to calculate the area in cm^3.

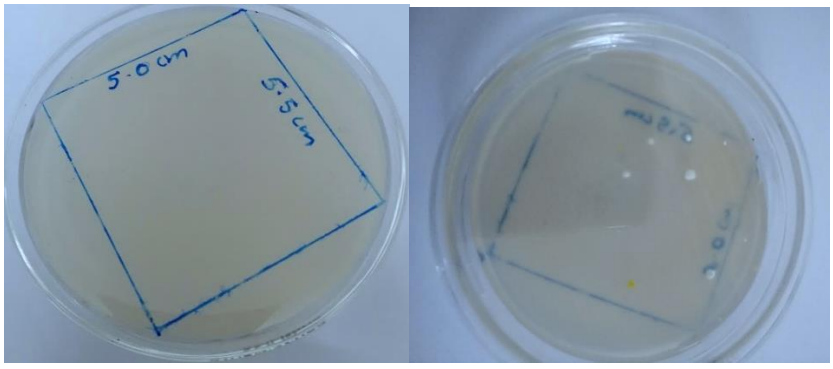

Figure 1. Earmarking on Microbial Growth Medium Plates to Count Microbial CFU/cm^3

The earmarked length on the plate was 5.5 cm with a breadth of 5 cm, and the height (air space available) on the growth medium on the plate was 1 cm, thus giving an exposed area of 27.5 cm^3. The microbial colonies of respective microbes *viz.* bacteria, fungi (*Mucor*, *Aspergillus*, *Fusarium*, sterile mycelia, *Pilobolus*, etc.), actinomycetes, and yeast contained in 27.5 cm^3 of air volume, which formed their respective colonies on growth media, were counted in the earmarked area. The number of colonies of a particular microbe obtained on this earmarked area was converted for m^3 area by employing the multiplication factor of 3.63 (100 divided by 27.5=3.63). Thus, the Colony Forming Unit (CFU) of each microbe was calculated as CFU/m^3 area.

2.2 Enumeration of Microbial CFU/Breath and Assessment of Microbial Air Quality Index

Conversion of m^3 to liter: 1 cubic meter is equal to 1000 liter, therefore an area of 27.5 cubic cm is equal to 0.0275 liters or 27.5 milliliters (air). During normal breathing an adult human breath around 500 mL air/breath which is known as tidal volume (Hallett et al., 2021), and therefore to convert 27.5 ml of air into 500 ml of air a conversion factor of 18 (500 divided by 27.5=18) was used. Thus, the number of microbial colonies obtained in a 27.5 cubic centimeter area was multiplied by 18 to get the number of CFU present per breath of air (i.e., 500 ml air).

2.3 Identification of Microbial Colonies

2.3.1 Identification of Bacteria

The bacterial colonies were studied for their morphology, color, elevation, shape, margin, and the gram reaction of the bacteria (Borkar, 2017). The prevalence of bacterial density/m^3 air was estimated.

2.3.2 Identification of Fungal Colonies

The fungal colonies were studied for their texture, and color and were identified under a binocular microscope based on the fungal spores, structures, and fruiting bodies (Funder, 1968).

2.4 In Vitro Evaluation of Triazole Antifungal Drug on Mucor Species

Ten isolates of *Mucor* species trapped on growth media from 10 locations of city air were studied for their reaction to two triazole antifungal drugs *viz.* Itraconazole (200 mg capsule) and Fluconazole (150 mg tablet), are being used for other invasive fungal infections in humans.

Different concentrations of these antifungal drugs *viz.* at 100 μg/mL; 500 μg /mL and 1000 μg/mL were tested against the mucor species isolates obtained from 10 different locations. Sterile PDA media containing the above concentrations of these triazole drugs were prepared, poured into sterilized Petri plates, and solidified. The 8 mm disc of the *Mucor* fungal growth of individual isolate was placed, in an inverted position, on these media in Petri plates. The inoculated plates were incubated in a BOD incubator at 29 ± 1^0C temp for *Mucor* growth/inhibition of *Mucor* growth, and the reading was taken after 5 days of incubation.

3. Results

3.1 Presence of Microbes in Nashik City Air at Different Locations

The microbes (Table 1) present and trapped in the Nashik city air were *Mucor*, *Aspergillus*, *Fusarium*, *Pilobolus*, and sterile fungi among the fungal species. Besides these fungal species, Actinomycetes and bacterial species were also present (Figure 2). No yeast/candida species were present in the air sample. On the air sample plates, the bacterial colonies appeared within 48 hrs; whereas the fungal colonies appeared after 5 days of incubation of the microbial growth media plates. Among the fungal species, *Mucor* was dominant over other fungal species with a range of 3.63 to 18.15 Colony Forming Units (CFU)/M^3 air and differed with the locations in the city (Figure 3). Maximum CFU/M^3 of *Mucor* was present in Panchavati, Bombay Naka followed by Trimbak road areas.

Table 1. Presence of Microbes in Nashik City Air at Different Locations

Name of location	Presence of microbes (*CFU*/M^3) in air						
	Mucor	***Aspergillus***	***Fusarium***	***Actinomycetes***	***Bacteria***	***Pilobolus***	**Sterile Fungi**
Dwarka	**3.63**	**0.0**	**0.0**	**29.04**	**119.79**	**0.0**	**0.0**
Deolali	**3.63**	**3.63**	**0.0**	**3.63**	**7.26**	**0.0**	**0.0**
Bytco Point	**3.63**	**3.63**	**3.63**	**3.63**	**29.04**	**0.0**	**0.0**
Pandav leni	**3.63**	**0.0**	**0.0**	**3.63**	**23.31**	**0.0**	**0.0**
Bombay Naka	**18.15**	**0.0**	**0.0**	**7.26**	**166.50**	**0.0**	**0.0**
Trimbak road (papaya nursery)	**10.89**	**0.0**	**0.0**	**3.63**	**96.57**	**0.0**	**0.0**
Panchavati	**18.15**	**0.0**	**0.0**	**21.78**	**153.18**	**0.0**	**0.0**
Adgao naka	**7.26**	**0.0**	**0.0**	**83.49**	**126.54**	**7.26**	**0.0**
Nandur naka	**7.26**	**0.0**	**3.63**	**7.26**	**29.97**	**0.0**	**0.0**
Mahsrud	**7.26**	**0.0**	**0.0**	**10.89**	**156.09**	**0.0**	**7.26**

The environment during air sampling: Air temp Max=35^0C; Min=29^0C, RH=77%, Time of sampling=3.00 PM.

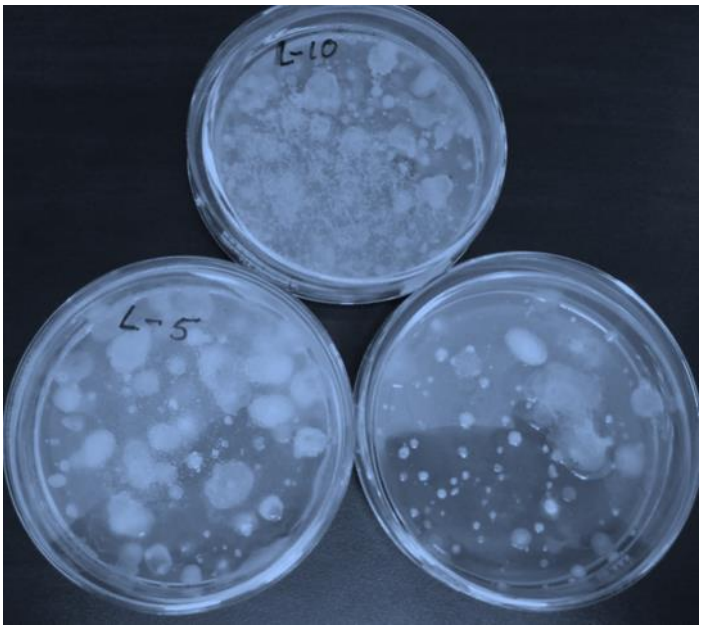

Figure 2. Microbial Flora of Nashik City Air

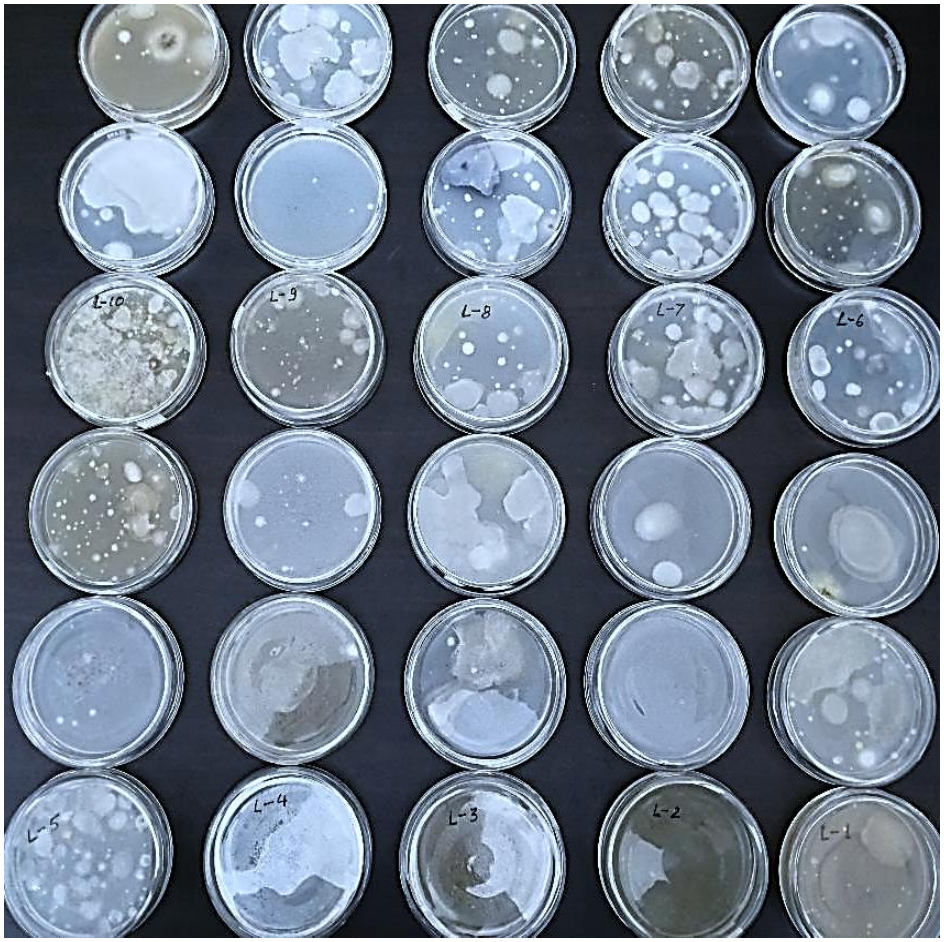

Figure 3. Trapping of Air Microbes at 10 Locations in Nashik City

The *Mucor* species trapped in the air sample exhibited the black color mycelial growth of the fungus (Figure 4) which was further purified and identified as *Mucor* sp. based on the fungal structures. The microscopic observations exhibited the black color sporangial fruiting bodies, sporangiospores, and aggregation of black-brown thick structures of mycelial masses as debris (Figure 5).

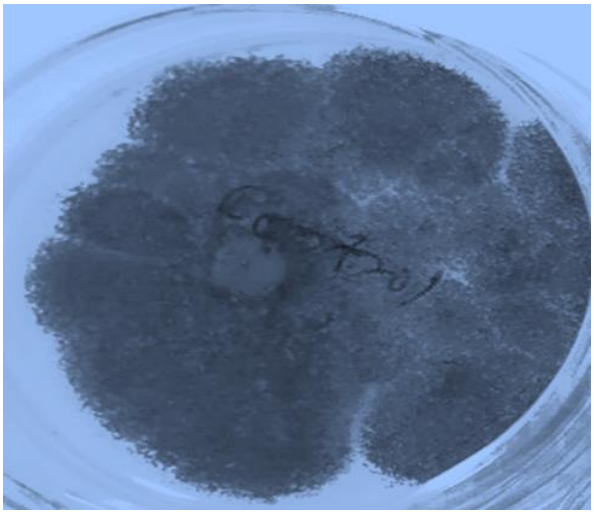

Figure 4. Fungal Colony of *Mucor* sp. Present in Nashik Air

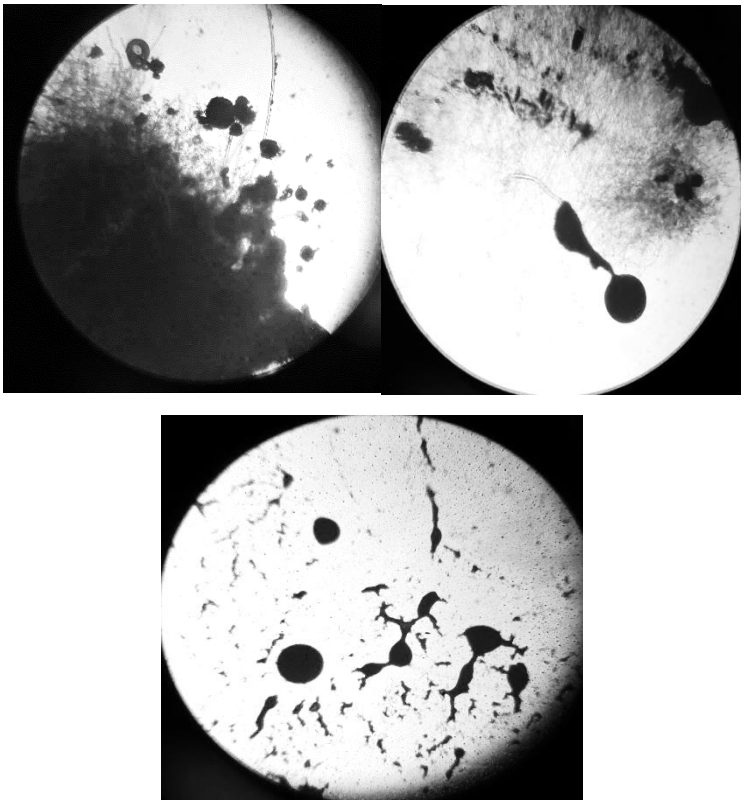

Figure 5. Microscopic Fruiting Bodies (Sporangium) of *Mucor* with an Aggregated Thick Mass of Fungal Debris on Growth Media and in Broth

The thick-walled fungal debris was more common besides the sporangium of the fungus in both solid (PDA) media and liquid potato dextrose broth. This fungal debris contained the mycelial and spore masses (Figure 6) of the *Mucor* fungus. Similar fungal structures are also reported in histopathological studies of mucormycosis infection (Figure 7) (Choudhary & Gahlot, 2021).

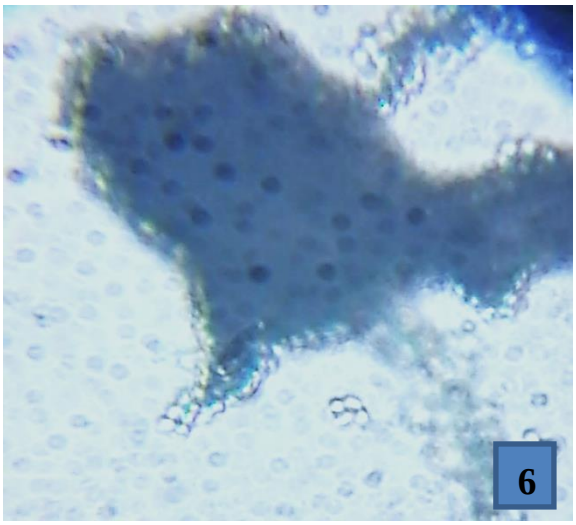

Figure 6. Spore Masses Turned into Black Thick Structures in Fungal Growth Media

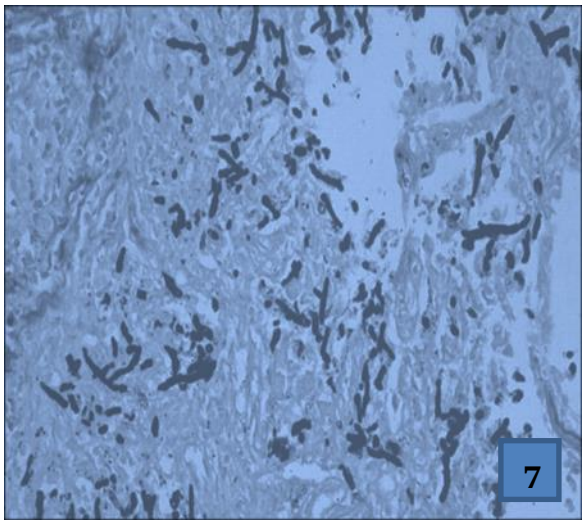

Figure 7. Aggregation of Fungal Hypha in Mucormycosis Infection

Among the air microbial communities, the bacterial species was most dominant and varies from 7.26 to 166.50 CFU/M^3 air. The maximum population of bacterial species was trapped in areas of Bombay Naka, Mahsrud followed by Panchavati. The bacterial species exhibited circular, raised, opaque, white, or pink colonies on growth media. The bacteria were gram-negative, cocci, and seem to be species of *Streptococcus*.

These fungal and bacterial species survived an atmospheric temperature of 35^0C during the May month of the summer season in the Nashik city air.

Mucor was the dominant species among the fungal microflora in the air. The fungal species load per tidal volume (the volume of air which we breathe in a single breath) was calculated for the city air (Table 2) which differed with locations in the city and for *Mucor* fungus, it ranged from 18 to 90.

Table 2. Presence of Fungal Microbes in the Tidal Volume of Air in Nashik City at Different Locations

Sr. no	Name of Location	Presence of fungal spores/breath (500mL air) in city air				
		Mucor	***Aspergillus***	***Fusarium***	***Pilobolus***	**Sterile Fungi**
1	**Dwarka**	**18**	**0.0**	**0.0**	**0.0**	**0.0**
2	**Deolali**	**18**	**18**	**0.0**	**0.0**	**0.0**
3	**Bytco Point**	**18**	**18**	**18**	**0.0**	**0.0**
4	**Pandav leni**	**18**	**0.0**	**0.0**	**0.0**	**0.0**
5	**Mumbai Naka**	**90**	**0.0**	**0.0**	**0.0**	**0.0**
6	**Trimbal road (Papaya nursery)**	**54**	**0.0**	**0.0**	**0.0**	**0.0**
7	**Panchavati**	**90**	**0.0**	**0.0**	**0.0**	**0.0**
8	**Adgao Naka**	**36**	**0.0**	**0.0**	**36**	**0.0**
9	**Nandur Naka**	**36**	**0.0**	**18**	**0.0**	**0.0**
10	**Mahsrud**	**36**	**0.0**	**0.0**	**0.0**	**36**

3.2 In Vitro Efficacy of Triazole Antifungal Drug against Mucor sp. Prevalent in Nashik City Air

The *in vitro* efficacy of two triazole antifungal drugs viz. Itraconazole and Fluconazole on the growth of mucor species indicated (Table 3) that these drugs were ineffective against the fungus. All three concentrations, i.e., 100, 500, and 1000 µg/mL were ineffective to check the growth and sporulation of the *Mucor* fungi (Figure 8). However, in the presence of these drugs, the mycelium has a reduced growth rate on the media containing these drugs, as compared to growth on normal growth media. On the normal growth media, the *Mucor* growth at 5 days was 7.00 cm diameter as against 1.45 to 2.77 cm on triazole-containing growth media. The percent inhibition of growth on triazole-containing media was 60.42 to 79.28 percent (Table 4).

Table 3. In Vitro Efficacy of Triazole Compounds against *Mucor* sp. Prevalent in Nashik City Air

Triazole compound	Concentration (µg /mL)	Mycelial growth (in cm) of location-specific *Mucor* isolate number										Average growth
		1	2	3	4	5	6	7	8	9	10	
Itraconazole (200 mg cap) containing PDA media	100	1.9	2.0	1.8	1.8	1.7	1.6	1.7	2.0	1.6	1.6	1.77
	500	1.7	2.0	1.5	1.9	1.4	1.5	1.6	1.8	1.7	1.7	1.66
	1000	1.2	1.5	1.5	1.7	1.4**	1.4	1.2	1.6	1.5	1.5	1.45
Fluconazole (150 mg tab) containing PDA media	100	2.4	3.0	2.0	2.0	2.5	2.9	2.6	1.9	4.0	4.0	2.77
	500	1.8	2.2	2.0	2.3	2.0*	2.0	1.8	1.8	2.6	2.6***	2.12
	1000	1.6	2.6	2.0	2.1	2.0***	2.4	1.9	1.5	2.0	2.4	2.05
Control PDA without Triazole compound	0.0	7.00										

Note. *=change in cultural characteristic; **=Fruiting structure of *Mucor* absent; ***=Reverse side of culture is pink-red as fungal growth absorbs Triazole compound to give pink-red color.

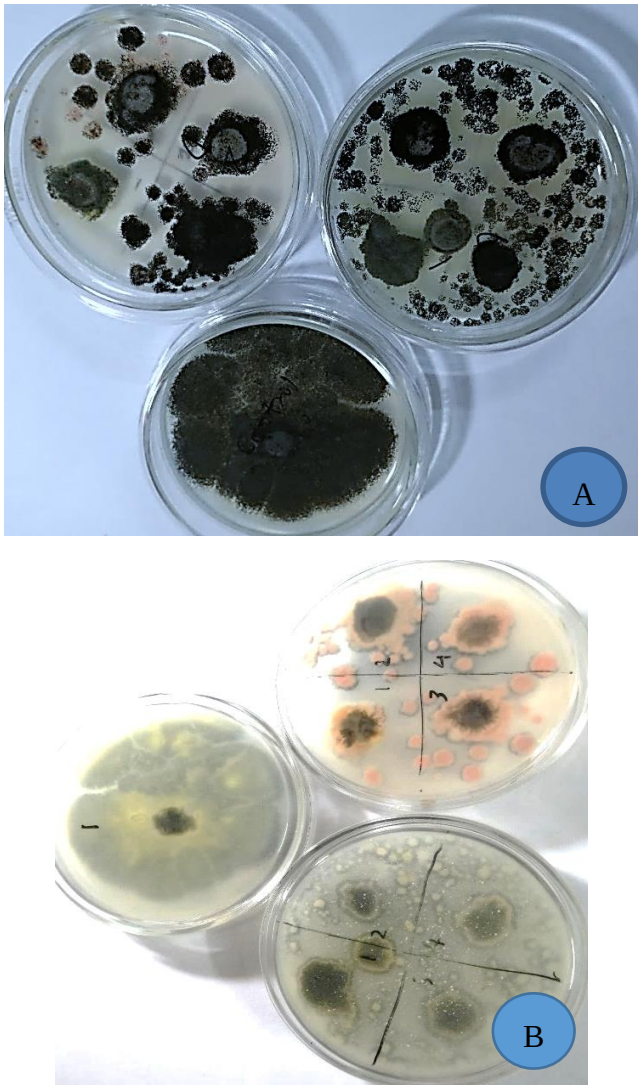

Figure 8. Growth of *Mucor* Isolates on Triazole Antifungal Drug Containing Growth Media (Front Side of Plate (A) and Reverse Side of Plate (B))

Table 4. Influence of Triazole Compounds on Growth Rate (cm/d) of *Mucor* sp. Prevalent in the City Air

Triazole compound	Concentration (ug /mL)	Average *Mucor* Growth * (in cm on 5th day)	Inhibition of *Mucor* growth (% inhibition)	Inhibition of growth rate (cm /d)
Itraconazole (200 mg capsule)	100	1.77	5.23 (74.71)	1.04
	500	1.66	5.34 (76.28)	1.06
	1000	1.45	5.55 (79.28)	1.11
Fluconazole (150 mg tablet)	100	2.77	4.23 (60.42)	0.84
	500	2.12	4.88 (69.71)	0.97
	1000	2.05	4.95 (70.71)	0.99
Control (No triazole compound)	0		7.00 cm growth on 5th day (growth rate 1.4 cm/d)	

Note. *=Average growth of 10 isolates of *Mucor* collected at different locations of the city, on particular triazole concentration.

4. Discussion

The fungus mucor is a causative agent of mucormycosis and mostly affects immunocompromised people. Several disease conditions like AIDS, Cancer, diabetes, malnutrition, certain genetic disorders, organ transplants, congenital immunodeficiency, primary immunodeficiency diseases (PDIs), lymphohematogenous malignancy (LHM), non-cytotoxic immunosuppression, splenectomy and chronic diseases with limited immune deficits are known as immunocompromised (Meidani et al., 2014) and may favor the infection of mucormycosis. The population of immunocompromised persons is estimated to be about 160 million people in the world (Anonymous, 2022) and thus this quantum of the population is in the risk group of mucormycosis, if the sufficient CFU of *Mucor* is present in a tidal volume to cause mucormycosis.

The presence of up to 90 CFU of *Mucor*/tidal volume seemed to be insufficient to cause mucormycosis, since no case of mucormycosis was reported in the city during the month of May 2022 and in subsequent months as compared to the mucormycosis cases in the same month in 2021. The probable

reason seems to be the hindrance of entry of *Mucor* spores by the cilia in the nostrils into the nasal cavities and paranasal sinuses and the innate immunity of the person. Mucormycosis is mainly a disease of the immunocompromised person (Pandilwar et al., 2020).

Thus, the study of microbial Air Quality Index (AQI) is an important issue while studying the air quality of cities and metros. The microbial air quality index can be defined as the sum of various CFU of microbes present in the air and their significance in causing the concern disease/diseases. This is the first publication on microbial AQI, mucor/tidal volume, and the methodology of its enumeration.

It is advised to maintain the air quality index for *Mucor* sp, a dominant fungal species in the city air at a minimal level to avoid mucormycosis infection. This can be achieved by employing the air vacuum cleaner machinery, in the *Mucor* species-dominated areas of the city based on the microbial air quality index for *Mucorales*.

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